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STUDIES ON THE EVOLUTION OF IMMUNOLOGIC UNRESPONSIVENESS FOLLOWING HAPTEN FEEDING*

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ABSTRACT

The establishment of immune tolerance after feeding a single large dose of picryl chloride to previously starved guinea pigs requires a minimal induction period of approximately 48 to 72 hours. Resistance to anaphylactic sensitization is regularly present at two days and reaches the highest levels by four days. In contrast, contact tolerance occurs sporadically at 72 hours and gradually increases in frequency to reach the maximum rate of inhibition over an interval of 18 to 21 days.

The timing of suppressing and challenging procedures is a crucial factor in the induction of immunologic unresponsiveness. However, the temporal evolution of tolerance to simple chemical antigens following oral administration has not been intensively studied because the regime most commonly employed to induce tolerance requires multiple feedings of the hapten over a period of weeks. This method makes it impossible to precisely define the point at which to begin measuring the length of time necessary for tolerance to evolve. The appearance of immunologic unresponsiveness in guinea pigs following ingestion of a single large dose of picryl chloride provides a method which permits direct measurement of time. Although not all aspects of tolerance following a single feeding have been studied, it is directly comparable to unresponsiveness established with multiple feedings (1). The development of both delayed contact hypersensitivity and antibody formation to hapten-protein conjugates is inhibited; the phenomenon is specific and the effect profound, although not absolute. The rate of unresponsiveness is dose related to a level beyond which increasing the amount of picryl fed does not increase the proportion of tolerant animals. Six months after the feeding, the capacity to respond is still suppressed in a majority of the animals (2).

In the following experiments guinea pigs were immunized at sequential intervals after hapten feeding and evaluated for the presence of immunologic unresponsiveness. The studies sought to investigate the effect of time on the frequency with which tolerance appears and to determine the optimal period required for maximal development of the phenomena.

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MATERIALS AND METHODS

Animals. Randomly bred male and female Hartley strain guinea pigs weighing 300 to 450 grams obtained primarily from BeauManor Farms, Cleveland, Ohio were used in these experiments.

Antigen. Picryl chloride (Eastman Organic Chemicals, Rochester, New York) was re-crystalized three or more times from a hot mixture of two parts absolute alcohol and one part benzol. The crystals were dried for several days in a desiccator over phosphorous pentoxide and then ground to a fine powder.

Feeding technique. The guinea pigs were intubated with a polyethylene tube as previously described and 1.5 ml of corn oil containing 60 mg of picryl chloride was slowly injected and then flushed with 0.25 ml of corn oil alone (1). This dose was selected because it proved to be as effective in depressing responsiveness as larger amounts (1).

Picryl conjugates. Picrylated bovine albumin—45 mg/gm of albumin and picrylated guinea pig serum—55 mg/gm of conjugate were prepared by the method of Benacerraf and Levine (3).

Tests for contact reactivity. Fifty microliters of 3 non-irritant concentrations of picryl chloride (1%, .3%, .1%) in acetone olive oil (4:1) were applied to the skin using a microsyringe. The sites were read at 24 hours and the intensity graded as follows: ± (trace) faint pink spots; + faint pink confluent macular erythema; ++ pink confluent macular erythema; +++ bright pink confluent macular erythema with a slightly thickened elevated edge; ++++ confluent bright pink erythema

with distinct thickening, elevation and necrosis. A 1+ or better reaction was considered a positive test.

Circulating antibody. Blood was obtained from the orbital sinus and the sera refrigerated or frozen for later testing by passive cutaneous anaphylaxis.

Passive cutaneous anaphylaxis (PCA). 0.1 ml of each serum was injected intradermally into the back of a normal guinea pig weighing 250 to 325 grams. Sixteen to eighteen hours later the animals were given an intravenous injection of 1 ml., 0.5% Evans blue (Matheson, Coleman and Bell, Cincinnati, Ohio) containing 5 mg. picryl bovine albumin (4). The blued sites were read at 30 minutes.

Immunologic unresponsiveness to contact sensitization. Active sensitization was attempted by the injection of 20 μg picryl chloride in complete Freund's adjuvant H$_{37}$RA (Difco Laboratories Inc., Detroit, Michigan) into each foot pad for a total of 80 μg. This procedure regularly produced contact reactivity and sometimes anaphylactic reactivity as well in the strain of guinea pigs used. Failure to develop contact dermatitis with an intensity of 1+ or better was interpreted to indicate immunologic tolerance to contact sensitization.

Detection of immunologic unresponsiveness to anaphylactic sensitization. A total of 5.6 μg of picrylated guinea pig serum was injected in complete Freund's adjuvant into the two rear foot pads. Two weeks later the animals were bled from the orbital sinus and the sera tested for the presence of circulating antibody to picryl bovine albumin by passive cutaneous anaphylaxis. This procedure regularly induced formation of circulating antibody to picryl protein conjugates and the absence of antibody was interpreted to indicate the presence of immunologic unresponsiveness to anaphylactic sensitization.

Experimental procedure. Day −1, solid food removed and guinea pigs permitted only water ad libitum; day 0, picryl chloride fed; day +1 solid food feeding resumed; days +1 to +21, active sensitization attempted at sequential intervals; 14 days after active sensitization—bleeding and contact tests; 15 days after active sensitization—contact tests read.

RESULTS

A. Temporal Evolution of Unresponsiveness to Contact Sensitization

The results of increasing the time interval between feeding and attempted sensitization on the rate and intensity of contact hypersensitivity in picryl-fed and control guinea pigs are shown in Table I. The picryl-fed animals immunized one day after feeding uniformly developed contact hypersensitivity to picryl chloride as did all the controls regardless of the time interval. In contrast, picryl-fed guinea pigs immunized more than one day following ingestion of the hapten failed to respond to antigenic stimulation in gradually increasing numbers. Inability to develop significant contact reactivity appeared in one animal after a latent period as brief as 72 hours, but more than seven days were necessary for a majority of the animals to become tolerant. The maximum rate was reached after an 18 day interval and allowing more time did not affect it.

Comparing the intensity of the positive reactions in the experimental and control groups reveals many 1+, but few 3 or 4+ responses in the sensitized picryl-fed animals, while con-

TABLE I

Results of increasing time intervals between and immunization on unresponsiveness to contact sensitization

Interval Between Feeding and Immunization	PICRYL FED		CONTROLS	
	Contact Reaction to 1% PCL		Contact Reaction to 1% PCL	
	Intensity of Reaction	Negative or Trace	Intensity of Reaction	Negative or Trace
1 Day	4+, 4+, 3+, 2+, 2+, 1+, 1+	0 (0%)	4+, 4+, 3+, 2+, 2+	0
3 Days	2+, 2+, 2+, 2+, 1+, Tr	1/7 (17%)	3+, 3+, 2+, 2+	0
5 Days	2+, 2+, 1+, 1+, 1+, Tr, 0	2/7 (29%)	4+, 4+, 4+, 3+, 2+	0
7 Days	4+, 3+, 1+, 1+, 1+, Tr. Tr	2/7 (29%)	4+, 4+, 4+, 3+, 2+	0
9 Days	1+, 1+, 0, 0, 0, 0, 0	5/7 (71%)	4+, 3+, 3+, 2+, 2+	0
11 Days	2+, 1+, 1+, 0, 0, 0, 0	4/7 (57%)	4+, 3+, 3+, 2+	0
14 Days	1+, 1+, 0, 0, 0, 0, 0	5/7 (71%)	4+, 4+, 4+	0
18 Days	3+, 0, 0, 0, 0, 0, 0	6/7 (86%)	4+, 4+, 4+, 3+	0
21 Days	1+, 0, 0, 0, 0, 0, 0	6/7 (86%)	4+, 4+, 3+, 3+, 2+	0

versely the contact tests in the corresponding controls all graded 2+ or better and 3 or 4+ responses were common. However, it should be pointed out that in previously reported experiments a few 1+ contact reactions occurred in control guinea pigs of the same stock immunized with the identical regimen (1).

The observation that, with one or two exceptions, the sensitized picryl-fed animals tended to have less intense contact reactions than their paired controls, implies the presence of partial or incomplete contact tolerance. Unfortunately this could not be confirmed because there was considerable overlapping and the small numbers preclude meaningful statistical analysis (5).

B. Temporal Evolution of Unresponsiveness to Anaphylactic Sensitization

Foot pad injections of picrylated guinea pig serum in adjuvant were used as the sensitizing challenge in these experiments because unconjugated picryl chloride failed to stimulate the formation of hapten-specific antibody with sufficient regularity. Table II shows that neither the control groups nor those immunized one day after feeding have a significant rate of anaphylactic unresponsiveness although a few of the controls did not make antibody. However, 71% of the animals immunized as briefly as 48 hours following feeding, were unable to respond. Similarly the majority of those immunized at later intervals up to 21 days, were also tolerant. The sera were not titered, but the positive PCA reactions in the control and experimental groups were of comparable size and intensity.

TABLE II

Effect of increasing time intervals between feeding and immunization on immunologic unresponsiveness to anaphylactic sensitization

INTERVAL	PCA ANTIBODY	
PICRYL FED	POSITIVE	NEGATIVE
1 Day	6/6	0/6 (0%)
2 Days	2/7	5/7 (71%)
4 Days	0/6	6/6 (100%)
7 Days	1/6	5/6 (83%)
14 Days	1/5	4/5 (80%)
21 Days	0/7	7/7 (100%)
Controls		
All Days	24/27	3/27 (11%)

DISCUSSION

These studies illustrate the temporal relationships of unresponsiveness induced by oral administration of a simple chemical to guinea pigs. Hapten feeding must precede the sensitizing attempt by more than a day to effectively suppress either contact or anaphylactic reactivity. In comparison, other tolerogenic procedures do not require an induction period and will depress responsiveness when performed after the sensitizing attempt. Inhibition of skin reactivity to neoarsphenamine has been observed in guinea pigs given an infusion of the chemical one day following a sensitizing intradermal injection of the same antigen (6, 7). Similarly drug induced immunosuppression produced by daily injections of cyclophosphamide or methotrexate will still effectively block contact reactivity to dinitrochlorobenzene or picryl chloride, even if the course is started as long as three to four days after the sensitizing procedure (8, 9). However, a response will appear when the drug is discontinued and paradoxically it has been shown that cyclophosphamide has a potentiating effect if the sensitizing attempt is performed on the last day of a five day course of the drug (10).

As the time between ingestion of the chemical and immunization is extended, resistance to contact or anaphylactic sensitization appeared with increasing regularity, but at differing rates. The frequency of contact tolerance rose gradually and required 18–21 days to reach maximal levels. This effect of hapten feeding on inhibition of contact hypersensitivity is similar to that reported by DeWeck and Frey, who found that the optimal period for the induction of contact tolerance to dinitrochlorobenzene in guinea pigs given dinitrochlorobenzene sulfonate intravenously was 14 to 28 days (11).

In contrast, the rate at which prior hapten feeding inhibited the capacity to form antibody rapidly reached maximal levels by four days and was sustained as the interval was lengthened. Similar studies on the rate at which anaphylactic unresponsiveness develops after infusion or feeding of hapten are not available for comparison. However, the kinetics of tolerance to antibody formation following the injection of protein antigens has been extensively investigated in

mice (12–14). Although these studies with protein antigens are more complex and not absolutely analogous, the results using a variety of antigens and systems, including cell transfer to remove cells from the environment of extra-cellular antigen, also indicate that a fairly short induction period, generally four to five days, is needed to establish tolerance to antibody formation. It is interesting to note that *in vitro* incubation with tolerogenic doses of antigen as brief as 15 minutes have been reported to result in a significant degree of tolerance in kinetic studies at a cellular level (15).

The rates at which contact and anaphylactic tolerance develop are not strictly comparable. However, even if partial contact tolerance is considered, the data suggest that inhibition of antibody formation occurs more rapidly and completely than suppression of contact reactivity. Perhaps during the induction phase of immunity, potential antibody forming cells are vulnerable to the tolerogenic effect earlier and/or in greater numbers than cells scheduled to mediate contact reactivity. It has been shown that use of sensitive techniques will detect antibody to the hapten NIP as early or earlier than contact reactivity, suggesting that antibody synthesis occurs more rapidly (16). To speculate further, the current concept is that tolerance occurs as a result of direct interaction between antigen and reactive lymphocytes (17). Ingested hapten first encounters lymphocytes in the gut wall and if the Peyers patches of gastrointestinal lymphoid tissue are viewed as the functional equivalent of the bursa of Fabricius in chickens —that is as an organ of lymphoid cells that populate the lymph nodes and differentiate into antibody forming plasma cells in contrast to thymus derived lymphocytes which are concerned with delayed type immunity—then it follows that antibody formation would be affected first (18). Additional experiments comparing the rates at which anaphylactic and contact tolerance appear following infusion of a hapten might indirectly help to clarify the role of gastrointestinal lymphoid tissue.

Whatever the series of events the tolerogen initiates following feeding, the finding that 48 hours or longer in the case of delayed reactivity is necessary for sensitization to be inhibited, indicates that exposure to antigen results in more than just a simple immediate destruction of potentially responsive cells. The role of persistent antigen is unclear. Ritts and Chase reported that most ingested picryl chloride is rapidly hydrolyzed to picric acid in the gut and were unable to locate any remaining hapten by autoradiography (19). Recent studies utilizing the sensitizing capacity of tissues from picryl chloride fed guinea pigs to detect residual hapten, have shown that elements of the upper gastrointestinal tract obtained up to 24 hours after ingestion, but no longer, have the ability to sensitize fresh animals (20). This indicates the amount of persistent hapten if any, is miniscule. These observations together with the absence of suppression at 24 hours provide substantial evidence that unresponsiveness even after feeding these large doses does not result from antigen depots absorbing antibody or reacting with sensitized cells.

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